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Derived amin acid sequences of the *nosZ* gene (respiratory N_2O reductase) from *Alcaligenes eutrophus*, *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* reveal potential copper-binding residues

Implications for the Cu_A site of N_2O reductase and cytochrome-*c* oxidase

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The *nosZ* genes encoding the multicopper enzyme nitrous oxide reductase of *Alcaligenes eutrophus* H16 and the type strain of *Pseudomonas aeruginosa* were cloned and sequenced for structural comparison of their gene products with the homologous product of the *nosZ* gene from *Pseudomonas stutzeri* [Viebrock, A. & Zumft, W. G. (1988) *J. Bacteriol. 170*, 4658–4668] and the subunit II of cytochrome-*c* oxidase (COII). Both types of enzymes possess the Cu_A binding site. The *nosZ* genes were identified in cosmid libraries by hybridization with an internal 1.22-kb *Pst*I fragment (NS220) of *nosZ* from *P. stutzeri*. The derived amino acid sequences indicate unprocessed gene products of 70084 Da (*A. eutrophus*) and 70695 Da (*P. aeruginosa*). The N-terminal sequences of the NosZ proteins have the characteristics of signal peptides for transport.

A homologous domain, extending over at least 50 residues, is shared among the three derived NosZ sequences and the Cu_A binding region of 32 COII sequences. Only three out of nine cysteine residues of the NosZ protein (*P. stutzeri*) are invariant. Cys618 and Cys622 are assigned to a binuclear center, *A*, which is thought to represent the Cu_A site of NosZ and is located close to the C terminus. Two conserved histidines, one methionine, one aspartate, one valine and two aromatic residues are also part of the Cu_A consensus sequence, which is the domain homologous between the two enzymes. The Cu_A consensus sequence, however, lacks four strictly conserved residues present in all COII sequences. Cys165 is likely to be a ligand of a second binuclear center, *Z*, for which we assume mainly histidine coordination. Of 23 histidine residues in NosZ (*P. stutzeri*), 14 are invariant, 7 of which are in regions with a degree of conservation well above the 50% positional identity between the *Alcaligenes* and *Pseudomonas* sequences. Conserved tryptophan residues are located close to several potential copper ligands. Trp615 may contribute to the observed quenching of fluorescence when the Cu_A site is occupied.

Copper, being the prosthetic metal of respiratory nitrous oxide reductase (N_2OR) and certain respiratory nitrite reductases, is essential for bacterial denitrification. N_2OR transforms N_2O to N_2 as the final step of the denitrification process. The first active enzyme was characterized from a marine strain of *Pseudomonas stutzeri* (Zumft and Matsubara, 1982; Coyle et al., 1985; Riester et al., 1989). Similar proteins were obtained from several other bacterial sources (for review see Zumft and Kroneck, 1990). Optical and EPR characteristics classify N_2O reductases within the same family but outside the realm of the classical types of copper proteins (Malkin and Malmström, 1970). A binuclear, mixed-valence center has been proposed to explain the 'anomalous' EPR features of N_2OR (Kroneck et al., 1988).

By generating a set of primary structures of N_2OR from different bacteria, we aimed to identify conserved regions with putative copper-coordinating residues as a basis for the directed exchange of ligands. *Alcaligenes eutrophus* and *Pseudomonas aeruginosa* were selected for this purpose, in addition to *P. stutzeri* from which the first sequence of a *nosZ* gene was determined (Viebrock and Zumft, 1988). By this choice, we hoped to find primary structures related neither too closely nor too distantly, to achieve a meaningful comparison. A polyclonal antiserum against N_2OR of *P. stutzeri* reacted with cell extracts from *P. aeruginosa*, but not with extracts from *A. eutrophus* (Körner et al., 1987). *P. aeruginosa* belongs with *P. stutzeri* to the well-defined rRNA homology group I (Palleroni et al., 1973) and to the 16S rRNA γ-3 subdivision of the purple bacteria (Woese, 1987). Within these groups, the

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Abbreviations. COII, cytochrome-*c* oxidase subunit II; ESEEM, electron-spin echo-envelope modulation; EXAFS, extended X-ray-absorption fine structure; MCD, magnetic circular dichroism; N_2OR, nitrous oxide reductase; Xaa, variable amino acid.

Enzymes. Nitrous oxide reductase (EC 1.7.99.6); ferrocytochrome *c*: oxygen oxidoreductase, cytochrome-*c* oxidase (EC 1.9.3.1).

Note. The novel nucleotide sequence data published here have been deposited with the EMBL/GenBank/DDBJ sequence data bank and are available under the accession numbers X65278 and X65277.

two organisms are rather distantly related. *A. eutrophus* H16 is a member of the 16S rRNA β subdivision of the purple bacteria. Its ability to denitrify is encoded on the 450-kb megaplasmid, pHG1, which also harbors genes for chemolithotrophy (Schneider et al., 1988). For *P. stutzeri*, there is no evidence that denitrification is a plasmid-encoded property.

Unexpected, but currently the most fascinating aspect of investigating N_2OR, is the finding that the EPR-active copper of the resting enzyme has properties similar to that of the Cu_A center of cytochrome-*c* oxidase (Jin et al., 1989; Scott et al., 1989; Kroneck et al., 1990; Antholine et al., 1992). In addition to the available spectroscopic evidence, there is a remarkable sequence similarity between the putative Cu_A binding site of subunit II of cytochrome-*c* oxidase (COII) and the C-terminal sequence of N_2OR (Viebrock and Zumft, 1988; Scott et al., 1989). This structural resemblance has been accepted by other w rkers (Covello and Gray, 1990; Buse and Steffens, 1991; Mather et al., 1991; Saraste et al., 1991), but to date was based on the derived gene product of only a single *nosZ* gene. Here we provide evidence for the conserved nature of the Cu_A site in N_2OR by isolating and sequencing two novel *nosZ* genes. Analysis of the gene products on the basis of the *P. stutzeri* sequence identifies a set of amino acid positions to be considered when modelling the copper-containing sites of N_2OR.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids

Sources for the *nosZ* genes were *A. eutrophus* H16, ATCC 17699 and *P. aeruginosa*, DSM 50071^T. The following strains of *Escherichia coli* were used as vector hosts: strain HB101 for plasmid pBR325 (Boyer and Roulland-Dussoix, 1969), strains JM105 (Yanish-Perron et al., 1985) and BMH 71 – 18 (Sambrook et al., 1989) for phage M13 and strain XL-1 Blue (Bullock et al., 1987) for the pBlueScript SKII(+) vector. For cloning procedures, the plasmid pBR325 (Bolivar, 1978), the cosmids pVK102 (Knauf and Nester, 1982) and pJA1 (Lindenmaier, 1985) and the phagemid pBlueScript SKII(+) (Alting-Mees and Short, 1989) were used.

M dia and growth conditions

Strains for DNA work were grown in Luria-Bertani medium. For solid media, 1.5% agar (Difco Laboratories) was added. *E. coli* strains were grown at 37°C, others at 30°C. Antibiotics were used at the following concentrations: ampicillin, 50 µg/ml; chloramphenicol, 30 µg/ml; kanamycin, 50 µg/ml; tetracycline, 10 µg/ml. Cell extracts from *A. eutrophus* and *P. aeruginosa* were prepared by high-pressure h mogenization of anaerobically nitrate-induced cells and clarified by ultracentrifugation before gel filtration.

DNA techniques

Genomic DNA was isolated as described by Meade et al. (1982). Standard procedures were used for isolation of plasmid DNA, endonuclease digestion, agarose-gel electrophoresis, dephosphorylation, ligation, preparation of competent cells and transformation (Sambrook et al., 1989).

Hybridization and cloning

The hybridization probe for screening, NS220, was an internal *Pst*I fragment of 1.223 kb of the *nosZ* gene from *P. stutzeri* (Viebrock and Zumft, 1988). This fragment was labeled with biotinylated 11-dUTP by nick translation (Rigby et al., 1977) and detected by a streptavidin – alkaline phosphatase conjugate (Leary et al., 1983) upon hybridization with the cosmid pGE26 of *A. eutrophus*. For colony hybridization of the *P. aeruginosa* gene library, the probe was labeled with 5'-[α-^{32}P]dCTP. Conditions for DNA hybridization and detection were as given by the Bethesda Research Laboratories Manual BlueGene™.

For cloning the *nosZ* gene from *A. eutrophus*, plasmid pHG1 was isolated and partially digested with *Hin*dIII (Eberz et al., 1986). A library of this plasmid was constructed in the cosmid vector pVK102. The cosmid pGE26 was identified by complementation of denitrification-negative mutants of *A. eutrophus*. It contained a 32.1-kb insert of three contiguous *Hin*dIII fragments of 5.1, 9.7 and 17.3 kb, respectively from pHG1. The restricted DNA of cosmid pGE26 was screened for the *nosZ* gene with the NS220 probe. The central 9.7-kb *Hin*dIII fragment of pGE26 was identified as the locus of the *nosZ* gene. This fragment was ligated into plasmid pBR325 to give plasmid pBR325-9.7. The restriction subfragments, termed ALC and used for sequencing, were obtained from this plasmid.

For cloning the *nosZ* gene of *P. aeruginosa*, a library was constructed in the cosmid vector pJA1 from a partial *Sau*3A digest of genomic DNA of *P. aeruginosa*. Ten clones that gave positive hybridization with the ^{32}P-labeled NS220 probe were propagated. From clone C6, a 2.8-kb *Eco*RI fragment was isolated and subcloned into the phagemid pBlueScript IISK(+) to yield plasmid pBS-E. Sequence analysis showed that the 3'-part of the *nosZ* gene was not located on this fragment. To obtain the complete *nosZ* gene, a 2-kb *Xho*I fragment, overlapping with the *Eco*RI fragment, was isolated from the same cosmid and subcloned into pBlueScript IISK(+) to yield plasmid pBS-X.

DNA sequence determination

The restriction subclones ALC1.3 (a 1.3-kb *Sal*I – *Hin*dIII fragment) ALC1.9 (a 1.9-kb *Pst*I fragment) and ALC1.6 (a 1.6-kb *Bam*HI – *Hin*dIII fragment) of pBR325-9.7 were generated in M13mp18 and M13mp19 vectors (Messing, 1983) and sets of nested deletions prepared by exonuclease-III treatment (Henikoff, 1984).

From plasmids pBS-X and pBS-E, various subclones were prepared in the vectors M13mp18 or pBlueScript SKII(+). Single stranded DNA was propagated from M13 derivatives in *E. coli* JM105 and BMH 71 – 18.

DNA was sequenced by the chain-termination method using deoxyadenosine-5'-[α-^{35}S]thiotriphosphate and Sequenase 2.0 (United States Biochemical Corp.). Compressions were resolved by using dITP in the sequencing mixture. In part, the DNA sequences were determined using sequence-derived primers, synthesized with an Applied Biosystems automatic DNA synthesizer. Both strands of the *nosZ* genes were sequenced. DNA and protein sequences were analyzed with the PC/GENE software (IntelliGenetics).

Chemicals

Radionucleotides were purchased from Amersham-Buchler. *Taq*-polymerase-based TaqTrack kit was from Pro-

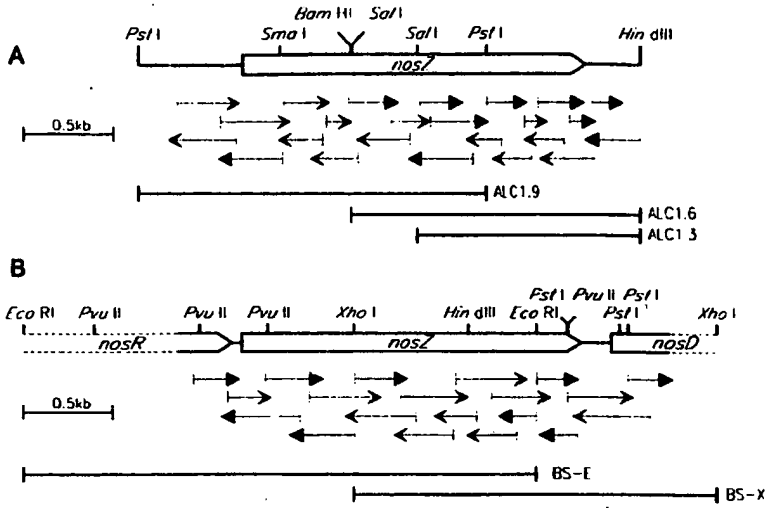

Fig. 1. Physical map, sequencing strategy, and open reading frames of the *nosZ* regions from (A) *A. eutrophus* and (B) *P. aeruginosa*. Open arrows show the direction of transcription of the indicated reading frames. The small arrows denote the sequenced parts of subclones generated from the ALC, BS-E and BS-X fragments. Clones generated by nested deletions and sequenced with the universal primer are represented by solid arrow heads, open arrowheads indicate clones which were sequenced by using sequence-derived primers.

mega. The kits for biotinylation of DNA by nick translation and DNA staining were from Gibco/BRL-Bethesda Research Laboratories GmbH. Restriction enzymes, calf intestinal alkaline phosphatase, T4 DNA ligase, Klenow fragment, exonuclease III and S1 nuclease were purchased from Boehringer GmbH.

RESULTS AND DISCUSSION

Primary structure of the *nosZ* genes

For the identification of the *nosZ* gene from *A. eutrophus*, a restriction map of the 9.7-kb *Hin*dIII insert of plasmid pBR325-9.7 was generated. The 1.3-kb *Sal*I – *Hin*dIII fragment, ALC1.3, was the smallest that still hybridized with the *nosZ* probe (Fig. 1A). By sequencing, it was shown that this segment encoded only the C-terminal half of the enzyme. The entire *nosZ* gene was localized on a 2.7-kb *Pst*I – *Hin*dIII fragm nt. Fig. 1A shows the restriction map of this part and the sequencing strategy used for *nosZ* of *A. eutrophus*. The established sequence comprises 2442 nucleotides (Fig. 2). The orientation of *nosZ* and the correct reading frame were inferred from the *nosZ* gene of *P. stutzeri* (Viebrock and Zumft, 1988). An unusual stretch of 26 nucleotides of G and C only, starting at position 340 (Fig. 2), could not be sequenced initially under a variety of conditions. Resolution was finally achieved by a combination of Sequenase, *Taq* polymerase and dITP (Khambaty and Ely, 1990).

To obtain the sequence of *nosZ* of *P. aeruginosa*, a restriction map of the cloned region and appropriate subclones were generated (Fig. 1B). A sequence of 2760 nucleotides was established (Fig. 3). The codon usage and the G+C content of *nosZ* from *P. aeruginosa* correspond to the characteristics of a chromosomal, non-pilin-like gene (reference data from West and Iglewski, 1988). The sequences flanking *nosZ* of *P. aeruginosa* were found to encode proteins previously identified in *P. stutzeri* (Fig. 4). In view of the apparent homology, sequencing of the flanking regions was limited to a short region of one strand, t establish the organization of the *nosZ* region in this bacterium (see Fig. 1B). The *nosZ* gene of *P. aeruginosa* is preceded by the regulatory gene *nosR* which is required for *nosZ* expression. A special feature of the derived *nosR* gene product is the presence of two cysteine clusters located close to the C terminus (Cuypers et al., 1992). This motif, suggested to be part of a redox-sensing mechanism, is conserved in the *P. aeruginosa* sequence (Fig. 4A). The *nosZ* gene is followed by *nosD*, the first of at least three genes located within an operon coding for copper-processing components specifically involved in the assembly of the copper chromophore(s) of N_2OR (Fig. 4B; Zumft et al., 1990).

The organization of the *nosZ* region of *A. eutrophus* remains unknown. Gene probes from the flanking regions of *nosZ* from *P. stutzeri* did not hybridize with restricted DNA from plasmid pGE26. The regions upstream and downstream of *nosZ* from *A. eutrophus*, as far as they were sequenced, did not reveal open reading frames with clear homology of their deduced products to NosR or NosD, respectively (Fig. 2).

The upstream, noncoding regions of the *nosZ* and the *nosD* genes harbor sequence motifs which are potential targets for the sigma factor σ^{54}, encoded by the *rpoN* gene (Figs 2 and 3). An *rpoN*-like gene was shown to control expression of denitrification in *A. eutrophus* (Römermann et al., 1989). Although Totten et al. (1990) have reported that an *rpoN*$^-$ mutant of *P. aeruginosa* still grows anaerobically on nitrate, the *nosZ* and *nosD* genes have motifs similar to the RpoN consensus sequence, $CTGGYAYRN_4TTGCAN_{6-11}N(+1)$ (Hunt and Magasanik, 1985), in their respective promoter regions. The anaerobic expression of the azurin gene from *P. aeruginosa* was suggested to be RpoN-dependent, from a putative binding motif in the promoter region (Arvidsson et al., 1989). Expression of N_2OR is regulated, among other factors, by oxygen. Concomittant with the finding of an *fnr*-like gene in *P. aeruginosa*, we have shown that several denitrification genes possess the binding motif for the FNR protein in their promoter region (Cuypers and Zumft, 1992). A motif corresponding to the canonical FNR-binding site, $TTGATN_4ATCAA$ (Spiro and Guest, 1990), with the exception of one nucleotide, is present upstream of *nosZ* in *A. eutrophus* (Fig. 2). No apparent FNR consensus was found for *nosZ* of *P. aeruginosa* concurring with *nosZ* of *P. stutzeri*,

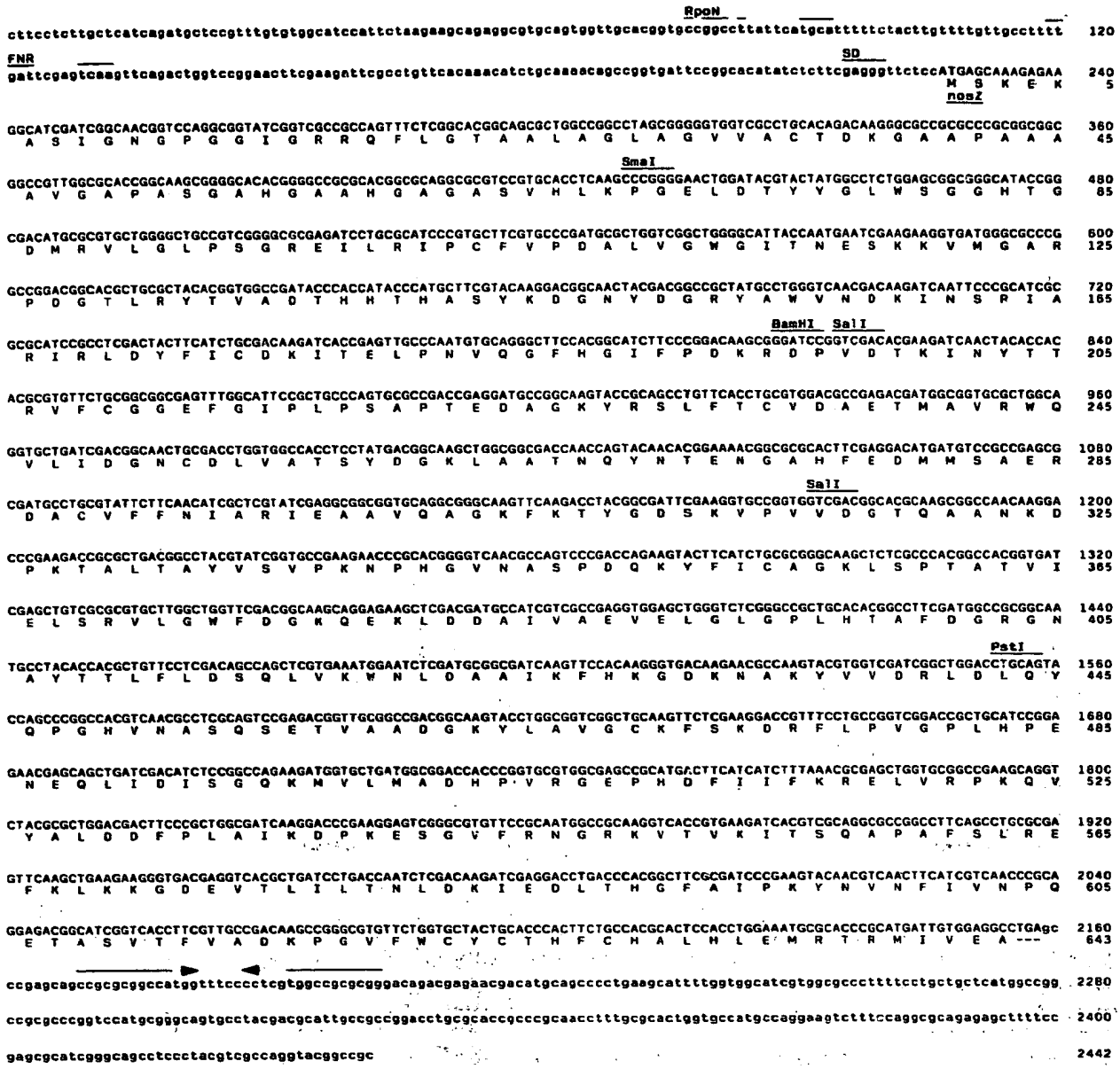

Fig. 2. Primary structure of the *nosZ* gene from *A. eutrophus*. Coding regions are in capital letters, intergenic noncoding regions in lower-case letters. The following regions are overlined and labeled: FNR-binding motif (FNR); putative RpoN-dependent promoter (RpoN); ribosome-binding site (SD); relevant restriction sites. The opposing arrows indicate a presumptive transcriptional terminator with a free energy of formation of -166.5 kJ mol^{-1} (Tinoco et al., 1973).

which has only a degenerated FNR motif upstream of the translational start. A detailed analysis will be required to establish the extent of involvement of σ^{54} and FNR in the expression of denitrification genes.

The *nosZ* genes have, immediately behind their respective stop codons, inverted repeats possibly functioning as rho-independent transcriptional terminators. *nosZ* of *P. stutzeri* shows a similar secondary structure and is transcribed as a monocistronic 2.25-kb mRNA (Cuypers et al., 1992). Since the genetic organization in the immediate vicinity of *nosZ* is identical in the two pseudomonads, it is likely that the *nosZ* gene of *P. aeruginosa* also represents a monocistronic transcriptional unit.

Comparative analysis of the NosZ proteins

The *nosZ* gene of *A. eutrophus* encodes a protein of 643 amino acids (M_r of 70084) and that of *P. aeruginosa* a product of 634 residues (M_r of 70695). Both proteins elute in gel filtration with an apparent M_r of approximately 140000, indicating that they are dimers (data not shown). The subunit mass of 73 kDa for N_2OR from strain P2 of *P. aeruginosa* (SooHoo and Hollocher, 1991) is reasonably close to the M_r of 65927, calculated from the primary structure of the type strain used in this work, assuming the processing of 46 residues (see below) but disregarding the contribution of copper to the protein mass. However, the amino acid composition of the

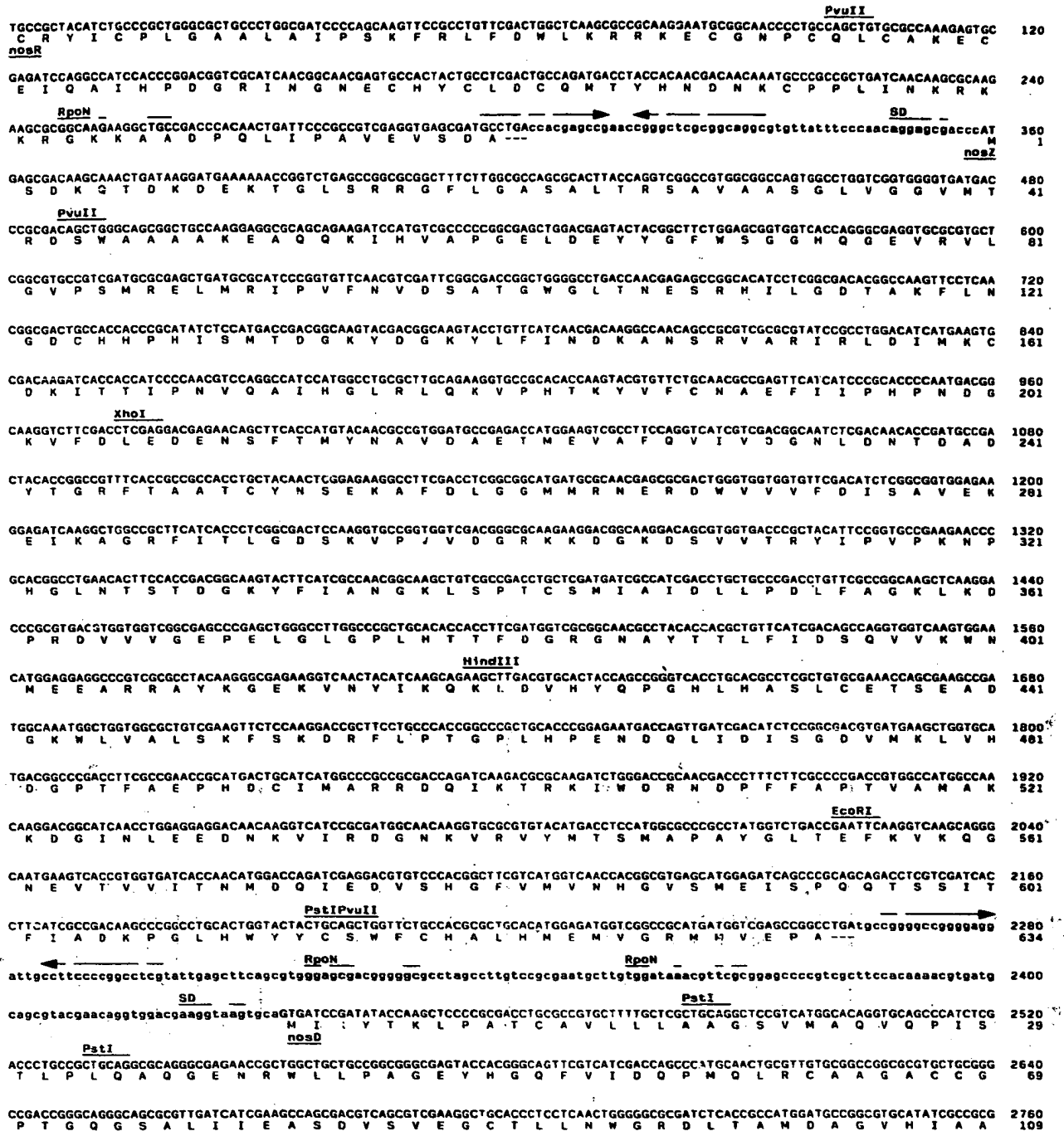

Fig. 3. Primary structure of the *nosZ* region from *P. aeruginosa*. The sequence, besides *nosZ*, covers the end of the *nosR* gene and the start of the *nosD* gene. The opposing arrows indicate presumptive transcriptional terminators with a free energy of formation for *nosZ* and *nosR* of -149.8 kJ mol^{-1} and 122.2 kJ mol^{-1}, respectively. Specific motifs in the sequence are labeled as in Fig. 2.

enzyme from strain P2 deviates by more than what could be ascribed to strain difference.

The N_2O reductases of *P. stutzeri* (Körner and Mayer, 1992) and other bacteria (for review see Zumft and Kroneck, 1990) are located in the periplasm. The N-terminal sequences of the deduced NosZ proteins of *A. eutrophus* and *P. aeruginosa* have the characteristics of a leader peptide, i.e. N-terminally located positive charges, a hydrophobic potentially membrane-spanning segment and several potential cleavage sites following the -1,-3 rule (von Heijne, 1986), which indicates that N_2OR is synthesized as a precursor for transport. The three leader sequences of NosZ have a common sequence motif, Gly-Xaa-Xaa-Arg-Arg-Xaa-Phe-Leu-Gly, that occurs in a similar form in leader peptides of the hydrogenase subunits from *A. eutrophus* (Friedrich, 1990; Friedrich, B., unpublished results). An alg rithm predicting transmembrane segments (Rao and Argos, 1986) indicates, for N_2OR from *A. eutrophus*, a membrane-anchor-like structure extending over Gly39 – Ala63, following the first possible cleavage site at Thr36. Since the currently known N_2O reductases have been

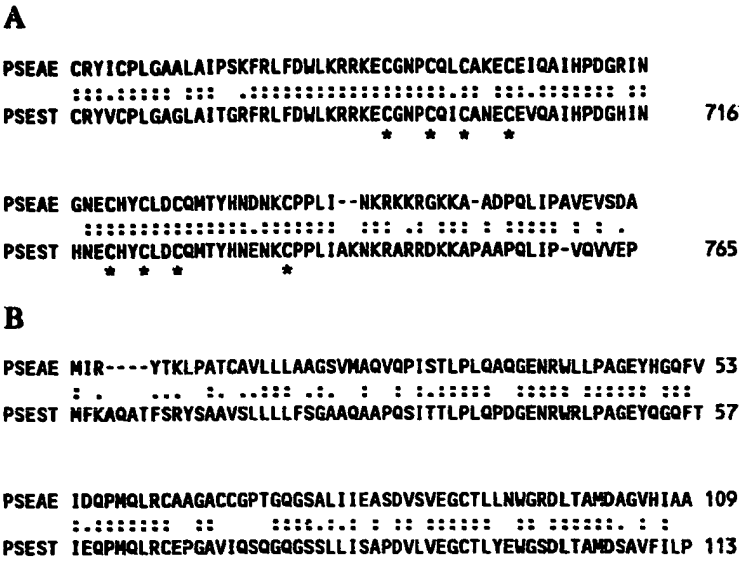

Fig. 4. Sequence alignment of the translated 5′-flanking and 3′-flanking regions of the *nosZ* genes from *P. aeruginosa* (PSEAE) and *P. stutzeri* (PSEST). (A) C-terminal part of NosR; several clustered cysteine residues with positional resemblance to bacterial ferredoxins are indicated by asterisks. (B) N-terminal part of the unprocessed NosD. Colons mark invariant residues, dots conservative amino acid replacements. Source of sequences: NosD of *P. stutzeri*, from Zumft et al., 1990; NosR of *P. stutzeri* (Cuypers et al., 1992).

reported to be soluble proteins (see Zumft and Kroneck, 1990), further studies are appropriate to define a possible membrane association of N_2OR in this bacterium.

The blocked N terminus of the *P. stutzeri* NosZ protein (Viebrock and Zumft, 1988) might be a pyroglutamyl residue at position 51 (Ser-Trp-Ala|Gln-Ala-Val), which corresponds to the Ser-Trp-Ala|Ala-Ala-Ala site of *P. aeruginosa* and results, for NosZ of this organism, in a leader peptide of 46 residues. NosZ proteins appear to have long signal sequences which are usually more typical of Gram-positive bacteria (von Heijne and Abrahamsén, 1989).

Sequence conservation

The derived amino acid sequences of NosZ from *A. eutrophus* and *P. stutzeri* have an overall positional identity of 50% (Fig. 5). There are clearly confined regions in the primary structure of the two reductases which have a degree of conservation well above average. The positional identity between *P. aeruginosa* and *P. stutzeri* is very high (89.9%) and limits the differentiation of functional regions for metal binding. The correspondence between the gene products of the two pseudomonads decreases to 80% (upstream) and 57.8% (downstream) in the regions flanking *nosZ* (Fig. 4). Unless specified otherwise, positional reference is made further on to the *P. stutzeri* sequence.

Fourteen out of 23 histidine residues of NosZ from *P. stutzeri* and 3 out of 9 cysteine residues are invariant in the three sequences (Fig. 5). Of those, His583, His623, His626, Cys618 and Cys622 belong to the Cu_A site. Position 165 is the only conserved cysteine within the N-terminal domain of the protein. Conserved methionine residues are at positions 227, 267, 268, 481, 629 and 633. By extended X-ray-absorption fine structure (EXAFS) analysis, several sulfur (S, Cl) and nitrogen (N, O) scatterers for copper were identified in resting and in reduced N_2OR (Scott et al., 1989; SooHoo et al., 1991). Currently, a two-center model accounts for most of the available spectrosc pic evidence for the copper sites of N_2OR. The paramagnetism observed in resting N_2OR is mainly attributed to a binuclear center, *A*. The contribution of a second paramagnet to this center is weak (Riester et al., 1989; Farrar et al., 1991; Antholine et al., 1992). The enzyme becomes EPR-silent on reduction by ascorbate. However, further reduction in the presence of phenazine methosulfate as mediator, or by dithionite, again generates a paramagnetic species which belongs to a presumably binuclear center, *Z* (Farrar et al., 1991; Jin et al., 1989; Riester et al., 1989). Resonance Raman features of the reduced center *Z* suggest a relationship with type-1 copper proteins and indirectly support a cysteine coordination (Dooley et al., 1987). The near-infrared bands in the circular dichroism spectra of N_2OR also indicate a highly covalent [Cu(II)S(Cys)] site (Dooley et al., 1991). The shape and intensities of the magnetic circular dichroism (MCD) spectra of oxidized and reduced N_2OR, although very diff r-ent from type-1 copper proteins, provide more evidence for a cysteine coordination in center *Z*. If this center is binuclear, a bridging cysteine residue would suffice to generate the two polarized charge-transfer transitions observed in the MCD spectrum (Farrar et al., 1991). It is likely that Cys165 represents this ligand, being the only conserved residue outside the Cu_A site. In its vicinity are the invariant histidines 129, 130, 132 and 178.

The electron-spin echo-envelope modulation (ESEEM), besides EXAFS, strongly indicates a multiple imidazole coordination to copper (Jin et al., 1989). The regions of the primary structure potentially providing these ligands may be circumscribed, considering those regions that show highly conserved neighborhoods as better candidates. Outside the Cu_A center these are histidines at positions 78, 326, 382, 433 and 467. They reside within regions (related to an undecapeptide centered at the histidine residue) ranging in positional identity over 63.6–90.9% between *A. eutrophus* and *P. stutzeri*. The highest degree of conservation is observed around His382 and His467. We assume that several of these histidines are part of center *Z*. Histidine residues at positions 61, 129, 130, 132, 178, 494 and 583, although conserved, are located in regions of a l w

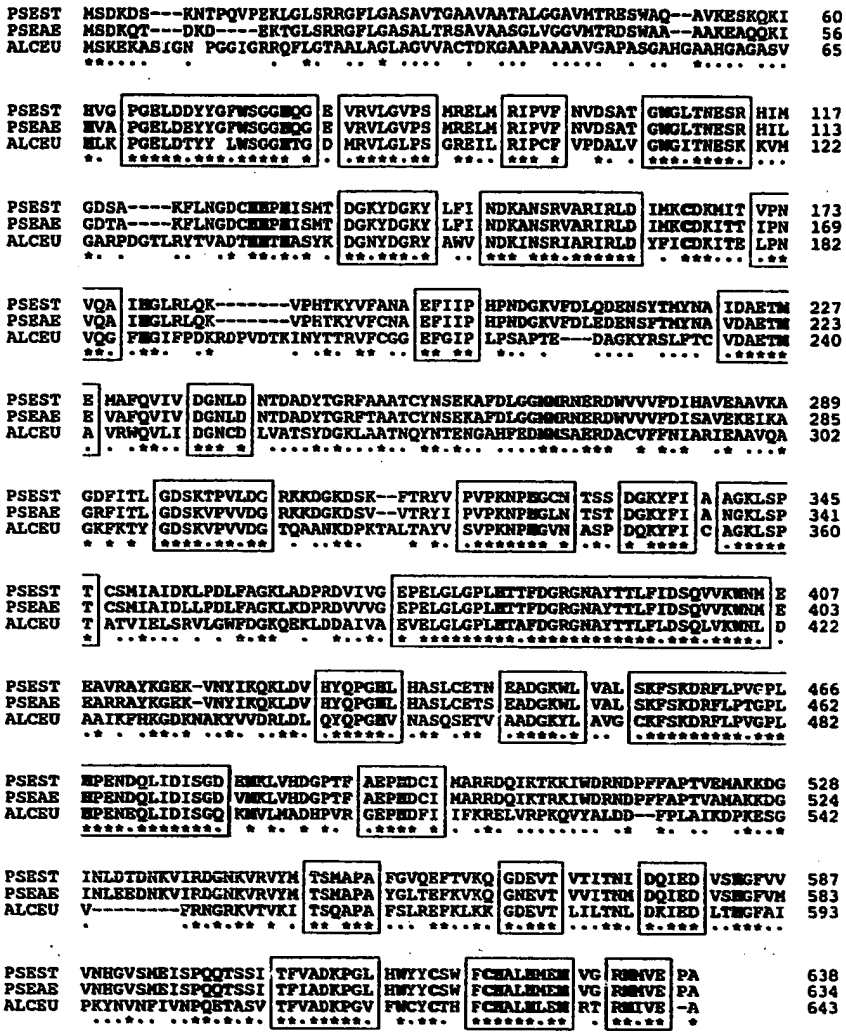

Fig. 5. Alignment of the derived amino acid sequences of the *nosZ* genes from *P. stutzeri* (PSEST), *P. aeruginosa* (PSEAE), and *A. eutrophus* (ALCEU). The multiple alignment of the NosZ sequences was performed by the program CLUSTAL (Higgins and Sharp, 1989); asterisks denote invariant residues, dots well-conserved residues. Regions in the sequence with 80% similarity within a window of five residues are boxed. The conserved cysteine, histidine, methionine and tryptophan residues are in boldface letters. The alignment was computed using the Dayhoff mutation data matrix with the following parameters: gap penalty 8, window size 16, filtering level 2.3, open gap cost 15 and unit gap cost 15. A lower gap cost only improves the alignment of the N-terminal residues with respect to the common sequence motif. Sequence source: NosZ of *P. stutzeri* from Viebrock and Zumft (1988).

sequence conservation (Fig. 5). However, His583 is part of the Cu_A domain, and the invariant cluster of three histidines, centered at His130, is followed by a longer stretch of conserved primary structure.

The NosZ protein has four conserved tryptophan residues (out of a total of nine in *P. stutzeri*). Trp76 lies within three residues of the invariant His78, closely followed by Trp107. Trp404 is part of the most strongly conserved region around His382, and Trp615 is a neighbor of Cys618 in the Cu_A site of N_2OR. Copper binding quenches the fluorescence of tryptophan, as shown by comparing the apoprotein with the enzyme low in copper from mutant MK402 and with resting N_2OR (Dooley et al., 1991). A tryptophan residue might, therefore, be part of, or sufficiently close to, a copper center to allow energy transfer from the aromatic residue to the metal site. It has previously been indicated that fluorescence quenching is better correlat d with an occupied Cu_A site than with the total copper content (Dooley et al., 1991). If this finding is substantiated, Trp615 within the Cu_A site may represent such a special residue for energy transfer (Fig. 5).

The Cu_A domain

The 50 C-terminal residues (Asp580 – Met629) of the three N_2OR sequences, when compared with the consensus sequence of COII, reveal, by cluster analysis (Higgins and Sharp, 1989), 12 positional identities and 16 well-conserved positions (Fig. 6). We extend here our previous proposal that this region forms a homologous Cu_A domain in N_2OR and COII because of its conservation of residues for metal binding and similar features with respect to the secondary structure of blue-copper sites. The inner limit of this domain may be confined by the conserved Pro164 of COII (*Paracoccus denitrificans*), whose corresponding residue in N_2OR (*P. stutzeri*) could be Pro553 which is shifted slightly upstream. A conserved aromatic region Trp-Xaa-(Tyr/Phe)-Xaa-Tyr in

the COII sequences (Buse and Steffens, 1991; Holm et al., 1987) is absent from N_2OR and, presumably, is not part of the common Cu_A domain. No overall sequence similarity is apparent between N_2OR and COII outside of this domain.

The alignment of the C-terminal parts of the three NosZ sequences with the COII consensus (the latter constituting the most-frequently occurring residues in the homologous region of 32 sequences of COII) yields the Cu_A consensus sequence Asp-(Val/Leu)-Xaa-His-Xaa-(Trp/Phe/Tyr)-Xaa_{29}-(Trp/Phe/Tyr)-Xaa_2-Cys-Xaa_3-Cys-Xaa_3-His-Xaa_2-Met (Fig. 6). Position 50 of the Cu_A consensus is a methionine (conserved both in N_2OR and COII), which disregards threonine at the same position of the soybean and rice sequences, but appears justified on the basis of mRNA editing of COII in plants (Covello and Gray, 1990). The sequence of *Paramecium primaurelia*, obtained from the database, was not considered because it contains an unidentified residue.

The seven-line EPR spectrum of resting N_2OR has been proposed to rise from a mixed-valence [Cu(1.5) ... Cu(1.5)] species (Kroneck et al., 1988). The spectrum simulation for frequencies ranging over 2.4–9.1 GHz yields consistent g values for N_2OR and cytochrome-*c* oxidase only when assuming a binuclear copper site, but not when considering a mononuclear site (Kroneck et al., 1990). ESEEM, EXAFS, multifrequency EPR and MCD data all show that the spectral features of N_2OR and cytochrome-*c* oxidase have remarkable similarities (Kroneck et al., 1988, 1990; Scott et al., 1989; Jin et al., 1989; Farrar et al., 1991; Antholine et al., 1992). This evidence led to the conclusion that cytochrome-*c* oxidase must have a copper site highly similar to that of N_2OR. That N_2OR can provide a valuable model for cytochrome-*c* oxidase rests on the facts that the enzyme from *P. stutzeri* has no heme moiety which, in cytochrome-*c* oxidase masks the signals of the Cu-Cu interaction, and that the copper of center *Z* is EPR-silent in the enzyme as isolated. As stated above, the contribution of a second paramagnet to center *A* is weak.

The Cu_A binding center of COII shows a relationship to the type-1 site of blue-copper proteins (Steffens and Buse, 1979; Holm et al., 1987). A His_2-Cys-Met coordination has been proven from crystal structures for the type-1 site (for review see Adman, 1991). The Cu_A site of cytochrome-*c* oxidase is also believed to be mononuclear, but has a His_2-Cys_2

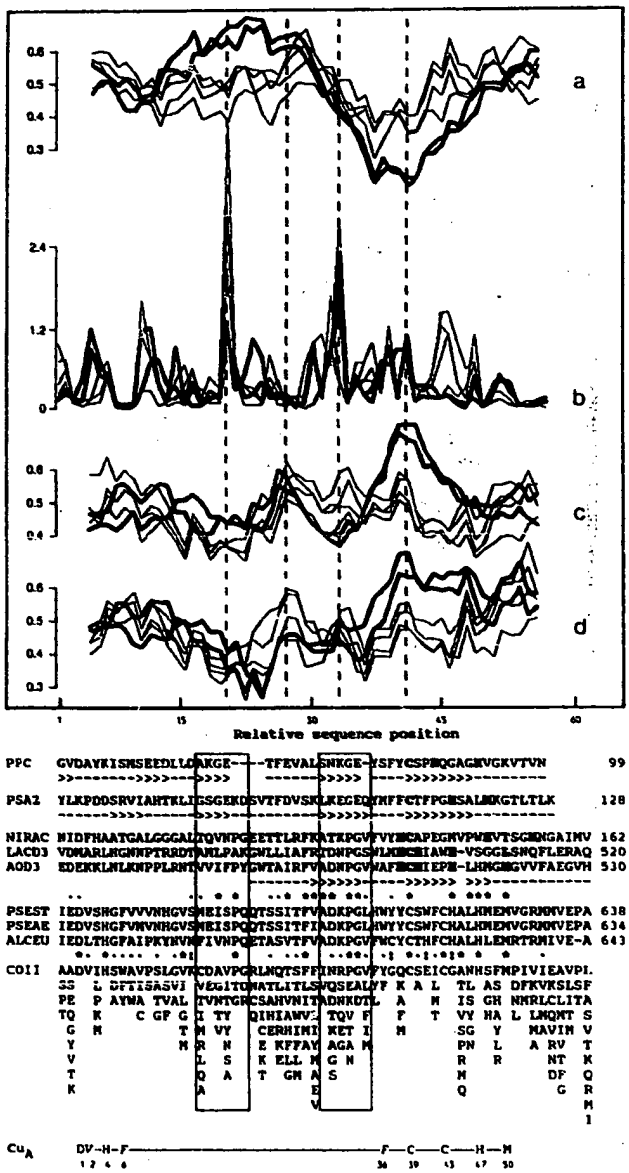

Fig. 6. Structural features of the Cu_A domain of N_2OR and COII, and of type-1 copper sites. The upper part shows superposition of profiles for the 60 C-terminal amino acids of N_2OR from *A. eutrophus* or 61 residues from *P. stutzeri* (heavy lines) and the corresponding region of COII sequences (thin lines) from *Pa. denitrificans* (Ala205–Gln265), yeast (Ala181–Leu241), bovine (Ser156–Leu216), and wheat (Pro184–Leu244). Profiles are (a) relative mutability of amino acids (Dayhoff et al., 1979); (b) probability of β-turns (Chou and Fasman, 1979); (c) conformational preference for antiparallel β-sheets (Lifson and Sander, 1979); (d) hydrophobicity profile using a contact-energy scale derived from crystal data (Miyazawa and Jernigan, 1985). The abscissae for the profiles a, c and d are normalized; the scale in b represents the turn probability $\times 10^4$. Sequence sources: COII (SWISS-PROT, release 18); *A. eutrophus* (this work); *P. stutzeri* (Zumft and Viebrock, 1988). The sequences in the lower half comprise the copper-binding regions of poplar plastocyanin (PPC; Guss and Freeman, 1983), azurin from *P. aeruginosa* (PSAZ; Canters, 1987), nitrite reductase from *Achromobacter cycloclastes* (NIRAC; Fenderson et al., 1991), rhus laccase domain 3 (LACD3; Germann et al., 1988), and cucumber ascorbate oxidase domain 3 (AOD3; Messerschmidt et al., 1989). They were aligned with the three N_2OR sequences (PSEST, PSEAE, ALCEU), and the COII consensus (labeled COII). β-Sheet is indicated by dashes, a turn region by arrow heads. The boxed parts emphasize the turn regions. Residues in boldface are proven ligands to copper. Invariant residues between the AOD3 and PSEST sequences are shown by asterisks, conservative replacements by dots. Multiple alignment comprising the COII consensus and the three NosZ sequences was made by the CLUSTAL program (see Fig. 4). The COII consensus and the variation found in 32 sequences, with the most frequently occurring amino acid in the top row and the other residues given in the order of decreasing frequency underneath each position, is shown in the lowermost part. The sources for the COII sequences are 29 entries from the SWISS-PROT databank (release 18; omitting *Pm. primaurelia*) and the sequences from *Bacillus subtilis* (Saraste et al., 1991), *Thermus thermophilus* (Mather et al., 1991), and bacterium PS3 (Ishizuka et al., 1990). A gap was disregarded in the COII consensus between position 34 and 35, that results from the six-residue insert in the sequences of *B. subtilis* and bacterium PS3. The double-headed arrows identify positions of absolute conservation in the COII consensus which are absent in N_2OR; asterisks denote invariant, dots well-conserved residues. The very last line gives the Cu_A consensus derived from N_2OR and COII. Phenylalanine (*F*) may be replaced by tyrosine or tryptophan; valine (*V*) is replaced by leucin in *A. eutrophus*. For further discussion, see the text.

coordination (Martin et al., 1988). If one accepts that N_2OR and cytochrome-*c* oxidase share a structurally related domain, coordination of Cu_A by histidine and cysteine only, is incompatible with the concept of a mixed valence, binuclear copper site, given the constraints from the primary structure of both enzymes.

A mixed-valence species does not necessarily require an entirely symmetrical coordination to allow an equal spin distribution over both copper atoms. A bridging ligand is assumed for this purpose, and a N(His)-S(Cys)-[Cu(1.5)-S(Cys)-Cu(1.5)]-N(His)-S(Met) coordination is considered for the Cu_A site (Antholine et al., 1992). In essence, this model combines elements of the type-1 copper site and the established minimal coordination of the Cu_A site of cytochrome-*c* oxidase with the constraint that only two conserved cysteine residues are available for this site (the third being part of the center *Z*). If the *bridging* ligand is a group other than cysteine (for instance carboxylate), each copper atom could have an individual cysteine ligand generating a coordination that would very likely be dominated by the two Cu-S(Cys) ligand-to-metal charge-transfer transitions. The Cu_A domain provides the set of core ligands required for the above model.

EXAFS of oxidized and reduced N_2OR, and of the mutant enzyme low in copper but with the Cu_A site occupied (Scott et al., 1989; Dooley et al., 1991), has been used to identify multiple Cu-(S, Cl) interactions. Among them is a long (approximately 0.26–0.27 nm) Cu-(S, Cl) distance that is tempting to view as a Cu-S(Met) interaction at the Cu_A center. The true Cu-S(Met) distance in the azurins from *Alcaligenes denitrificans* (Shepard et al., 1990) and *P. aeruginosa* (Nar et al., 1991) is approximately 0.32 nm and, therefore, much longer. We note, however, that using EXAFS, this bond in the latter protein had been estimated to be only 0.27 nm (Groeneveld et al., 1986). In poplar plastocyanin, the Cu-S(Met92) distance is 0.29 nm (discussed by Guss et al., 1986).

Several common features can be identified within the Cu_A domain. Fig. 6 shows superimposed profiles for the relative mutability of amino acids, predictions for β-turns and β-sheet and hydrophobicity profiles for four representative COII sequences from prokaryotes and eukaryotes, as well as for two NosZ proteins. Despite the disparity between organisms and two types of enzymes, similar patterns are apparent for the Cu_A region. The variability of residues in the COII sequences has two broad minima around positions 4 (His) and 39–43 (Cys-Xaa_3-Cys) of the Cu_A consensus sequence (a few exceptions are also noticeable). These minima coincide with a decrease in the relative mutability of amino acids, particularly around the Cys-Xaa_3-Cys motif (Fig. 6). Further similarity is observed when extending the comparison to the structure of the type-1 site of copper proteins. In the blue-copper proteins, the copper binding site follows a large loop which crosses the 'south' of the protein (Gus and Freeman, 1983; Adman, 1991). The β-turn structure preceding this loop seems to be conserved in the Cu_A domain. The residues Asp580 and His583 (*P. stutzeri*), equivalent to positions 1 and 4 of the Cu_A consensus, respectively, are conserved both in N_2OR and COII, which suggests essentiality of these residues. It has been proposed that these residues are brought into the proximity of the Cys-Xaa_3-Cys motif (Cys618 and Cys622 of *P. stutzeri*) to serve as ligands (Holm et al., 1987). Depending on the exact composition of the Cu_A site, further histidines could also be provided by a second protein domain, as observed for the trinuclear copper center of ascorbate oxidase. We find that the Cu_A site of N_2OR shows certain sequence similarity to the copper-binding domain 3 of this oxidase (Fig. 6). Domain 3 of ascorbate oxidase harbors the type-1 copper center and contributes four histidines to the trinuclear copper center bonded between domain 1 and 3 (Messerschmidt et al., 1989).

The Cu_A site, initially believed to be unique to cytochrome-*c* oxidase, is clearly present in N_2OR. MCD studies are in pr gress to further support this conclusion (Bingham et al., 1991). Of course, it should not be implied that the Cu_A binding sites of N_2OR and COII are identical in every aspect. Not surprisingly, the alignment shows one lysine, one glutamate, and two glycine residues conserved in the COII consensus sequence but not in N_2OR (Fig. 6). The set of conserved residues differing in the Cu_A domain of N_2OR and COII may reflect the catalytic activity of the two enzymes, related to the interaction with specific electron donors or different intramolecular electron transfer pathways. In cytochrome-*c* oxidase, the Cu_A center is seen either as an integral part of the proton-translocating mechanism (Gelles et al., 1986) or as a structurally rigid-metal site for fast-electron transport, similar to the blue, β-sheet copper proteins (Williams, 1987; Holm et al., 1987). The three N_2O reductases structurally investigated so far are soluble enzymes and there is no evidence that they would contribute to proton translocation other than by providing an electron sink for the anaerobic respiratory chain. The question which needs to be answered is what are th functional specificities of a binuclear Cu_A center compared to a type-1 site? Wilson and Bickar (1991) suggested, for cytochrome-*c* oxidase, the possibility that a $2H^+/e^-$ stoichiometry is accomodated at a binuclear site, but a strong case has been made recently for Cu_B as the redox-active element in proton pumping (Babcock and Wikström, 1992). Thus, the question remains open as to why this peculiar metal site is present in two types of respiratory enzymes reducing O_2 or N_2O. Evolutionary aspects appear as an attractive rationale (Zumft, 1992), but will only become more convincing if a functional basis can be found.

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